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FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER **P22,816A USA** U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5) 10/018030
INTERNATIONAL APPLICATION NO. **PCT/US00/09178**	INTERNATIONAL FILING DATE **April 6, 2000**	PRIORITY DATE CLAIMED **April 30, 1999**

TITLE OF INVENTION

Variants of TRAF2 which Act as an Inhibitor of TNF-Alpha (TNFα) Signaling Pathway

APPLICANT(S) FOR DO/EO/US

George H. Searfos III, Marco F. Pagnoni, Yuri D. Ivashchenko, Kun Guo and Kenneth L. Clark

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). (unexecuted)
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Sequence listing; computer diskette; Sequence Certification; and Fee Calculation Sheet (in duplicate)

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5) 10/018030	INTERNATIONAL APPLICATION NO. PCT/US00/09178	ATTORNEY'S DOCKET NUMBER P22,816A USA

24. The following fees are submitted:.	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
☒ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$100.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	32 - 20 =	12	x $18.00	**$216.00**	
Independent claims	10 - 3 =	7	x $84.00	**$588.00**	
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$904.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$904.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$904.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$904.00**	
				Amount to be: refunded	$
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a. ☒ A check in the amount of **$904.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ________ in the amount of ________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **19-5425** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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10/018030

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October 26, 2001

IN THE UNITED STATES RECEIVING OFFICE
AS THE DO/EO OF THE PATENT COOPERATION TREATY

Re: George H. Searfoss III, Marco F. Pagnoni, Yuri D. Ivashchenko, Kun Guo and Kenneth L. Clark
U.S. National Phase Application
Based on Intl. Application No. PCT/US00/09178
Filed April 6, 2000
Variants of TRAF2 which Act as an Inhibitor of TNF-Alpha (TNF α) Signaling Pathway

Attorney Docket No. P22,816-A USA

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail Post Office to Addressee, Mailing Label No. EL663032275US, in an envelope addressed to Commissioner for Patents, Box Patent Application, Washington, DC 20231 on October 26, 2001.

Mike B. Lavelle

Commissioner for Patents
Box Patent Application
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Before commencing examination of the above-identified application, please amend the application as follows.

In the Specification

Page 1, line 1, after the title, insert the following:

--**CROSS REFERENCE TO RELATED APPLICATIONS**

This application is a U.S. national stage application based on International Application No. PCT/US00/09178, filed April 6, 2000, which claims priority to U.S. Provisional Application No. 60/131,940, filed April 30, 1999.--

Respectfully submitted,

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VARIANTS OF TRAF2 WHICH ACT AS AN INHIBITOR OF TNF-ALPHA (TNF α) SIGNALING PATHWAY

Field of the Invention

Tumor necrosis factor α (TNF α) is an intercellular mediator of immune responses produced by a variety of cells, including activated macrophages and monocytes. The responses triggered by TNF α are initiated through its interaction with two distinct TNF α cell surface receptors: TNFαR1 and TNFαR2. TNF α binds to these cell surface receptors and triggers activation of transcriptional factors, for example, nuclear factor κB (NFκB), which regulate the expression of a variety of immune and inflammatory response genes.

Upon the binding of TNF α, the TNF α receptors interact through their cytoplasmic domains with a variety of intracellular signal translation proteins. One group of intracellular signal translation proteins known to associate with the TNF α receptors are the tumor necrosis factor receptor associated factors known as the "TRAF" family of receptor proteins. The TRAF family is comprised of a number of homologous proteins which share common structural features and which associate with and transduce signals from TNF α receptor proteins. The TRAF proteins lack enzymatic activity motifs and instead appear to function as adapter proteins which couple the receptors to downstream signaling cascades.

One member of the family, TRAF2, associates with a number of TNF α receptor family proteins, including TNFαR1, TNFαR2, *CD40 and CD30. For TRAF2, direct binding of at least eight* intracellular molecules has been identified. TRAF2 has been shown to be critical for TNF α mediated activation of a variety of transcriptional factors, in particular, NFκB and the C-jun N-terminal kinase (JNK/SAPK) and these transcription factors are in turn responsible for expression of an immune/inflammatory response.

There are a variety of disease states that are linked to regulatory pathways controlled by TNF α binding. In some instances, TNF α binding triggers an inflammatory response which ultimately results in a disease state. Accordingly, it would be desirable to develop means for preventing diseases related to TNF α receptor binding. In particular, it would be desirable to find a way to prevent activation of an inflammatory response that would otherwise be initiated by TNF α activation. The present invention provides polypeptides which are based on TRAF2 and which are capable of inhibiting the TNF α signaling pathways in order to treat and prevent diseases linked to TNF α binding.

Reported Developments

The general structure of the TRAF proteins has been described and is illustrated generally in Figure 1(a) which shows in diagrammatic form full-length TRAF2 (TRAF2-FL). These proteins have an N-terminal region with a zinc ring finger motif, followed by an array of zinc finger-like structures. The zinc finger region is followed by a conserved (TRAF) domain which is composed of two subdomains: an N-terminal domain and a C-terminal domain. The C-terminal domain is involved in receptor association and homo-, as well as hetero-oligomerization of TRAFs, and serves as a docking site for a variety of other signaling proteins.

TRAF2 follows the general structure of the TRAF proteins described above. A number of studies have attempted to

correlate the structural subdomains of the TRAF2 protein with the protein's functions.

Takeuchi et al. performed extensive mutational analysis on TRAF2 (Takeuchi et al., *J. Biol. Chem.*, 271(33) 19935-42 (1996)). These studies suggest TRAF2 is composed of modular domains mediating distinct activities. The authors determined that the N-terminal ring finger and 2 adjacent zinc fingers of TRAF2 are required for NFκB activation and that the distinct TRAF-N and TRAF-C subdomains within the TRAF domain appear to independently mediate self association and interaction with TRAF1.

Song et al. (*Proc. Natl. Acad. Sci. USA*, 94, 9792-9796 (1997)) followed up on studies showing that the TNF α induced activation of NFκB and the c-jun N-terminal kinase (JNK/SAPK) requires TRAF2. The authors showed that TRAF2 is the bifurcation point of two kinase cascades leading to activation of NFκB and JNK. This observation supports a functional model for TRAF2 and other members of the TRAF family as adaptor proteins with docking sites for additional signaling proteins that initiate parallel downstream responses.

Min et al. (*J. Immunology*, 159, 3508-3518 (1997)) used a transfection/overexpression strategy to analyze the roles of TRAF proteins. TRAF2 containing the TRAF domain, but lacking amino terminal residues 1-80 had been previously shown to inhibit TNF α induced NFκB activation. The authors demonstrated that this TRAF2 variant also blocked JNK activation by TNF α.

Brink et al., (*J. Biol. Chem.*, 273, 7, 4129-4134 (1998)) described a splice variant of TRAF2 which they called "TRAF2A." The cDNA of TRAF2A is identical to TRAF2 with the exception of an extra 21 bp of sequence encoding a seven amino acid insert within the TRAF2A ring finger domain. The authors found that TRAF2A mRNA expression is regulated in a tissue specific manner and TRAF2A protein is capable of

binding to the cytoplasmic domain of TNFαR2. They also found that, in contrast to TRAF2, TRAF2A is unable to stimulate NFκB activity when overexpressed in 293 cells and acts as a dominant inhibitor of TNFαR2 dependent NFκB activation.

Many studies have linked inflammatory processes and TNFα with the major cardiovascular disease states (Bryant et al., *Circulation*, 97(14):1375-81 (1998); Kubota et al., *Circ. Res.*, 81(4):627-35 (1997); Muller Werdan et al., *Eur. Cytokine Netw.*, 9(4):689-91 (1998); Aukrust et al., *Am. J. Cardiol.*, 83(3):376-82 (1999)). Over the past five years, evidence has accumulated which indicates that raised local TNF α levels are associated with: (a) cardiac ischemia-reperfusion injury which follows myocardial infarction, coronary artery bypass surgery, cardiac transplantation or ischemia-reperfusion injury in the CNS following stroke; (b) the progression and rupture of advanced coronary atherosclerotic plaques; (c) the development and progression of congestive heart failure; and (d) endothelial cell injury following balloon angioplasty. In addition, recent findings suggest that apoptotic cell death may be an important factor in the pathophysiology of myocardial cell death during heart failure or infarction. It is known that TNF α can induce myocyte apoptosis.

In addition to the cardiovascular disease states mentioned above, there are a variety of other disease states whose pathogenesis is linked to TNF α. These disease states include Crohn's disease, psoriasis, rheumatoid arthritis, graft versus host disease, inflammatory bowel disease, non-insulin dependent diabetes and neurodegenerative diseases (e.g., Parkinson's disease).

Given the relationship between TNF α and a large variety of diseases such as those discussed above, it would be desirable to provide compositions and methods for inhibiting and treating these disease states.

Summary of the Invention

In accordance with the present invention, it has been found that variants of TRAF2, in particular, a variant that includes a naturally occurring splice variation (TRAF2TR) and a variant that includes the naturally occurring splice variation and a deletion in the N-terminal region of TRAF2 (TRAF2TD), provide for inhibition of TNF α signal transduction and the associated immune inflammatory responses.

In accordance with one embodiment of the present invention, there is provided a DNA sequence encoding TRAF2TR comprising the sequence as shown in Figure 2a.

In accordance with another embodiment of the present invention, there is also provided a DNA sequence encoding TRAF2TD comprising the sequence as shown in Figure 3a.

In a preferred embodiment, the TRAF2TR and TRAF2TD DNA are cDNAs.

In other embodiments, the present invention provides a TRAF2TR polypeptide which is capable of inhibiting tumor necrosis factor α (TNF α) regulated pathways comprising an amino acid sequence as shown in Figure 2b and a TRAF2TD polypeptide which is capable of inhibiting TNF α regulated pathways comprising an amino acid sequence as shown in Figure 3b.

Another aspect of the present invention provides a method of inhibiting TNF α regulated pathways in a patient comprising introducing into the body of the patient a composition which is capable of inhibiting the TNF α regulated pathway and which comprises an expression vector capable of expressing TRAF2TR polypeptide, an expression vector capable of expressing TRAF2TD polypeptide, a TRAF2TR polypeptide and a pharmaceutically acceptable carrier, or a TRAF2TD polypeptide and a pharmaceutically acceptable carrier.

Still another aspect of the present invention provides a method of inhibiting diseases involving overproduction of TNF α comprising administering to a patient a composition which is capable of inhibiting TNF α regulated pathways and which comprises an expression vector capable of expressing TRAF2TR, an expression vector capable of expressing TRAF2TD, a TRAF2TR polypeptide and a pharmaceutically acceptable carrier, or a TRAF2TD polypeptide and a pharmaceutically acceptable carrier.

Yet another aspect of the present invention provides a method of inhibiting TNF α pathologies involving hyperactivation of nuclear factor κB(NFκB) dependent genes comprising administering to a patient a composition which is capable of inhibiting TNF α regulated pathways and which comprises an expression vector capable of expressing TRAF2TR, an expression vector capable of expressing TRAF2TD, a TRAF2TR polypeptide and a pharmaceutically acceptable carrier, or a TRAF2TD polypeptide and a pharmaceutically acceptable carrier.

The present invention also provides a method of inhibiting inflammatory processes involving tumor necrosis factor α comprising administering to a patient a composition which is capable of inhibiting TNF α regulated pathways and which comprises an expression vector capable of expressing TRAF2TR, an expression vector capable of expressing TRAF2TD, a TRAF2TR polypeptide and a pharmaceutically acceptable carrier, or a TRAF2TR polypeptide and a pharmaceutically acceptable carrier.

In certain embodiments, the inflammatory process is selected from the group consisting of Crohn's disease, psoriasis, rheumatoid arthritis, graft versus host disease, inflammatory bowel disease, non-insulin dependent diabetes and neurogenerative diseases.

In yet another embodiment, the inflammatory process is a cardiovascular disease selected from the group consisting of

(a) cardiac ischemia-reperfusion injury following myocardial infarction, coronary artery bypass surgery, cardiac transplantation or ischemia-reperfusion injury in the CNS following stroke; (b) the progression and rupture of advanced coronary atherosclerotic plaques; (c) the development and progression of congestive heart failure; (d) endothelial cell injury following balloon angioplasty; and (e) apoptotic cell death of myocardial cells.

In yet another embodiment of the present invention, there is provided a DNA sequence encoding a TRAF2TR/2TD variant.

Another aspect of the present invention provides a TRAF2TR/2TD variant polypeptide which is capable of inhibiting TNF α-regulated pathways.

The present invention provides the advantage of being able to treat a wide variety of disease states using variants of a naturally-occurring protein which interferes with an early event common to these disease states, i.e., TNF α signal transduction.

Brief Description of the Drawings

Figure 1 is a schematic structure of full length TRAF2 (TRAF2-FL) and the alternatively spliced variant, TRAF2TR.

Figures 2a and 2b are the nucleic acid sequence (2a) of TRAF2TR cDNA and the amino acid sequence of TRAF2TR (2b).

Figures 3a and 3b are the nucleic acid sequence of TRAF2TD (3a) and the amino acid sequence of TRAF2TD (3b).

Figures 4a and 4b are the nucleic acid (4a) and amino acid (4b) alignment of spliced TRAF2 (TRAF2TR) and full length TRAF2.

Figure 5 illustrates the tissue distribution of TRAF2TR variant mRNA. Lanes: 1 - control TRAF2FL cDNA; 2 - control

TRAF2 spliced variant (TRAF2TR) cDNA; 3 - Jurkat; 4 - HeLa cell line; 5 - Thymus; 6 - placenta; 7 - Thymus; 8 - spleen; 9 - ovary; 10 - control TRAF2FL.

Figure 6 illustrates the immunodetection of Myc-fused TRAF2FL and TRAF2TR in transfected HeLa cells. Lanes: 1-pcDNA3 vector; 2-myc-TRAF2FL; 3-myc-TRAF2TR.

Figure 7 illustrates an electrophoretic mobility shift assays (EMSA) that was performed using an NFKB UAS probe. Nuclear extracts from cells overexpressing FL TRAF2 (lanes 3 and 4) show TNF-alpha induced shifts significantly stronger in comparison to control (lanes 1 and 2). TRAF2-TR overexpression blocks formation of NF-kB and, as a result, no shift has been detected in TNF α stimulated cells (lanes 5 and 6).

Detailed Description of the Invention

There are set forth hereafter definitions of terms used herein and descriptions of preferred embodiments of the present invention.

Definitions

A "cloning vector" is a replicon, for example, a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control. A cloning vector may be capable of replication in one cell type and expression in another ("shuttle vector"). In preferred embodiments of the present invention, the cloning vector is capable of expression in a host cell and the "expression vector" is able to express TRAF2TR or TRAF2TD at sufficient levels to interfere with a TNF α regulated pathway in the cell.

A "cassette" refers to a segment of DNA that can be inserted into a vector at one or more specific restriction

sites. The segment of DNA encodes a polypeptide of interest and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. The transforming DNA can be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or of deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") or of any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term "nucleic acid molecule" and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, for example, a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the

appropriate conditions of temperature and solution ionic strength (*see* Sambrook et al., *infra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 18 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding TRAF2. Oligonucleotides can be labeled, *e.g.*, with ^{32}P-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid encoding TRAF2. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of TRAF2, or to detect the presence of nucleic acids encoding TRAF2. In a further embodiment, an oligonucleotide can form a triple helix with a TRAF2 DNA molecule. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, etc.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The DNA coding sequences and the appropriate regulatory sequences are preferably provided in an expression vector. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is

not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as, for example, promoters, enhancers, and terminators that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then spliced (if the coding sequence contains introns) and translated into the protein encoded by the coding sequence.

As used herein, the term "homologous" refers to the relationship between proteins that possess a "common evolutionary origin." Such proteins (and their encoding genes) have sequence homology, as reflected by their high degree of sequence similarity. The term "sequence

similarity" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin. However, in common usage and as used herein, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

The term "TNF α regulated pathway" and related terms refer to signal transduction pathways involving the binding of TNF α to a member of the tumor necrosis factor receptor family (TNFR).

The term "corresponding to" is used herein to refer to similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity and not the numbering of the amino acid residues or nucleotide bases.

The term "splice variant" refers to a polypeptide encoded by an mRNA produced by alternative processing of the full length mRNA encoded by a gene or genes resulting in an mRNA that contains one or more deletions relative to the full length mRNA for the genes.

__Embodiments of the Invention__

The present invention relates to two variants of TRAF2 which inhibit TNF α signaling pathways. One embodiment is an RNA processing splice variant of TRAF2 referred to hereinafter as "TRAF2 truncated" or "TRAF2TR". Another embodiment is based on TRAF2TR having a deletion of amino acid residues 1 to 87 relative to TRAF2TR and is referred to as "TRAF2 truncated-deleted" or "TRAF2TD". Both TRAF2TR and TRAF2TD have the ability to inhibit TNF α signaling pathways. TRAF2TD is a particularly preferred embodiment due to its ability to dramatically reduce the response to TNF α binding.

There follows hereinbelow a description of the structure of these two embodiments, followed by a discussion on how to prepare these embodiments.

Structure and Preparation of TRAF2TR Embodiment

The cDNA sequence for this splice variant is presented in Figure 2a and the amino acid sequence is presented in Figure 2b. Referring to Figure 1 which shows TRAF2TR schematically, it can be seen that the deletion removes amino acid residues 123 to 201 of TRAF2FL, which encompasses the C-terminal portion of Zn finger domain 1 and all of the Zn fingers 2 and 3, as well as the N-terminal residues of Zn finger 4.

The TRAF2TR embodiment of the present invention can be prepared by any suitable method, including a variety of methods known to those of skill in the art. Teachings on the isolation, cloning and sequencing DNA can be found in a variety of sources. General molecular biology, microbiology and recombinant DNA techniques within the skill of the art, are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Given the information in the description herein on the DNA sequence of TRAF2TR and the known methods in the art for obtaining cDNA, nucleotide sequences encoding TRAF2TR and TRAF2TD can be cloned readily or prepared from wild type TRAF2 and inserted into an appropriate vector for expression

of these proteins *in vitro* or *in vivo*. For a description of methods relating to cloning cDNA and expression vectors, see Sambrook et al., 1989, *supra*.

A gene encoding TRAF2, whether genomic DNA or cDNA, can be isolated from a human genomic library or cDNA library. Methods for obtaining a gene given the DNA sequence information presented herein are well known in the art. The TRAF2 DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"). It is obtained preferably from a cDNA library prepared from tissues with high level expression of the protein (*e.g.*, cells of lymphoid origin, in particular, B cells or an osteosarcoma cell line, for example, human osteosarcoma SAOS-2 (ATCC No. HTB-85) that exhibit high levels of expression of TRAF2 or TRAF2TR). The DNA may also be obtained by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (See, for example, Sambrook et al., 1989, *supra*; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II) or by chemical synthesis. Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. Given that the present invention is based in part on the isolation of a splice variant (TRAF2TR) of full length TRAF2, it is desirable to obtain a cDNA encoding the TRAF2TR sequence.

Methods for obtaining cDNA are well known in the art. Briefly, these methods include isolating a mixture of messenger RNA (mRNAs) from eukaryotic cells and employing a series of enzymatic reactions to synthesize double-stranded DNA copies (cDNAs) complementary to the isolated mRNAs.

It has been found that reverse transcriptase-polymerase chain reaction (RT-PCR) cloning is an efficient way to isolate cDNA containing the TRAF2TR splice variant as presented in Example 1 hereinbelow. RT-PCR involves reverse transcription of cellular mRNA with the enzyme reverse

transcriptase followed by subjecting the resultant DNA product to amplification using PCR.

Regardless of the method used to obtain the desired cDNA, the double-stranded cDNA mixture is inserted into cloning vehicles by any one of many known techniques, depending at least in part on the particular vehicle used. Various insertion methods are discussed in considerable detail in *Methods in Enzymology*, 68, 16-18 (1980), as well as in Sambrook et al., 1989, *supra*.

Once the DNA segments are inserted into a cloning vehicle, the cloning vehicle is used to transform a suitable host. These cloning vehicles usually impart an antibiotic resistance trait on the host. Such hosts are generally prokaryotic cells and only a few of the host cells contain the desired cDNA. The transfected host cells constitute a gene "library", providing a representative sample of the mRNAs present in the cell from which the mRNAs were isolated.

Given the sequence information on TRAF2 provided herein, an appropriate oligonucleotide sequence may be prepared, preferably synthesized as discussed above, and used to identify clones containing TRAF2 sequences. To identify clones containing the TRAF2 sequences, individual transformed or transfected cells are grown as colonies on a nitrocellulose filter paper. The colonies are lysed and the DNA is bound tightly to the filter paper by heating. The filter paper is then incubated with a labeled oligonucleotide probe which is complementary to TRAF2. DNA fragments with substantial homology to TRAF2 will hybridize to the probe. The greater the degree of homology, the more stringent hybridization conditions can be used.

The probe hybridizes with the cDNA for which it is complementary. It can be identified by autoradiography or by chemical reactions that identify the presence of the probe. The corresponding clones are characterized in order to identify one or a combination of clones which contain all of

the structural information for the desired protein. The nucleic acid sequence coding for the protein of interest is isolated and reinserted into an expression vector. The expression vector brings the cloned gene under the regulatory control of specific prokaryotic or eukaryotic control elements which allow the efficient expression (transcription and translation) of the ds-cDNA.

Further selection can be carried out on the basis of the properties of the gene. For example, if the gene encodes a protein product having the isoelectric, electrophoretic, amino acid composition, or partial amino acid sequence of the TRAF2 protein as disclosed herein. Thus, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones can be selected which produce a protein that has similar or identical properties to TRAF2TR with regard to electrophoretic migration, isoelectric focusing, non-equilibrium pH gel electrophoresis, proteolytic digestion, or antigenicity.

<u>Structure and Preparation of TRAF2TD Embodiment</u>

Relative to TRAF2TR, TRAF2TD has a deletion of amino acids 1 to 87 and the corresponding nucleotides encoding these amino acids. The DNA sequence for TRAF2TD is presented in Figure 3a and the amino acid sequence is presented in Figure 3b.

Any suitable method can be used to prepare the TRAF2TD embodiment, including, for example, a variety of methods based on the information provided above. In particular, there are a number of methods for creating a truncated version of TRAF2TR containing a deletion of amino acids 1 to 87. In a preferred method of preparation, TRAF2TR cDNA is used as a template for PCR using a 5' primer encompassing nucleotides 262 to 280 of the TRAF2 full length coding sequence (ATGAGTTCGGCCTTCCCAGAT wherein the ATG codon was included to create a translation initiation site; the 3'

primer was TTA TAG CCC TGT CAG GTC CAC. The resulting construct begins at amino acid 88 of full length TRAF2 and contains the 123 to 201 amino acid deletion of TRAF2TR.

Additional variants of TRAF2TR can be prepared using methods such as those described above for the preparation of TRAF2TD.

__TRAF2TR/2TD Variants__

The present invention includes within its scope allelic variants, substitution, addition and deletion mutant variants, analogs, and derivatives of TRAF2TR or TRAF2TD (hereinafter referred to as "TRAF2TR/2TD variants") and homologs from other species that have the same or homologous functional activity as TRAF2TR. In preferred embodiments, genes having deletions or substitutions that increase the ability to inhibit TNF α signaling pathways are utilized in the practice of the invention. Preparation or isolation of TRAF2TR/2TD variants are within the scope of the present invention. Accordingly, the scope of the present invention includes TRAF2TR/2TD variants which are functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with TRAF2TR.

TRAF2TR/2TD variants can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, TRAF2TR/2TD embodiments are made that have enhanced or increased functional activity relative to TRAF2TR or TRAF2TD.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as TRAF2TR, including an amino acid sequence that contains a single amino acid variant, may be used in the practice of the present invention. These include, but are not limited to, allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of TRAF2TR which are altered by the substitution of different

codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the TRAF2TR/2TD variants of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a TRAF2TR protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free $CONH_2$ can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential

site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (*i.e.*, His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

The genes encoding TRAF2TR/2TD variants of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned TRAF2 gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a TRAF2TR/2TD embodiment, care should be taken to ensure that the modified gene remains within the same translational reading frame as the TRAF2TR gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the TRAF2TR/2TD-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo* to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated TRAF2TR gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, *J. Biol. Chem.* 253:6551; Zoller and Smith, 1984, *DNA* 3:479-488; Oliphant et al., 1986, *Gene* 44:177; Hutchinson et al., 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83:710), use of "TAB" linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in PCR Technology: Principles and Applications for DNA

Amplification, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

The discussion which follows relates to the manipulation and expression of DNA encoding the desired polypeptides is common to both TRAF2TR and TRAF2TD, as well as TRAF2TR/2TD variants.

Cloning of TRAF2TR, TRAF2TD and TRAF2TR/2TD Variants into Cloning/Expression Vectors

The identified and isolated DNA sequence can be inserted into an appropriate cloning/expression vector (hereinafter "vector") to facilitate modifications to the sequence or expression of the protein. These vectors typically include multiple cloning sites, promoters, sequences which facilitate replication in a host cell and selection markers.

Any suitable vector can be used. There are many known in the art. Examples of vectors that can be used include, but are not limited to, plasmids or modified viruses. The vector is typically compatible with a given host cell into which the vector is introduced to facilitate replication of the vector and expression of the encoded proteins. The insertion of a DNA sequence into a given vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Useful vectors may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Examples of specific vectors useful in the practice of the present invention include, but are not limited to, *E. coli* bacteriophages, for example, lambda derivatives, or plasmids, for example, pBR322 derivatives or

pUC plasmid derivatives, *e.g.*, pmal-c, pFLAG, derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col E1, pCR1, pMal-C2, pET, pGEX (Smith *et al.*, 1988, *Gene* 67:31-40), pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage 1, e.g., NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast vectors such as the 2 μm plasmid or derivatives thereof; vectors useful in eukaryotic cells, for example, vectors useful in insect cells, such as baculovirus vectors, vectors useful in mammalian cells; vectors derived from combinations of plasmids and phage DNAs, plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Yeast vectors that can be used according to the invention include, but are not limited to, the non-fusion pYES2 vector (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Gst*XI, *Eco*RI, *Bst*XI, *Bam*H1, *Sac*I, *Kpn*1, and *Hind*III cloning sit; Invitrogen) or the fusion pYESHisA, B, C (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Bst*XI, *Eco*RI, *Bam*H1, *Sac*I, *Kpn*I, and *Hind*III cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen).

Baculovirus vectors that can be used in the practice of the invention include a variety of vectors, including both non-fusion transfer vectors, for example, pVL941 (*Bam*H1 cloning site; Summers), pVL1393 (*Bam*H1, *Sma*I, *Xba*I, *Eco*R1, *Not*I, *Xma*III, *Bgl*II, and *Pst*I cloning site; Invitrogen), pVL1392 (*Bgl*II, *Pst*I, *Not*I, *Xma*III, *Eco*RI, *Xba*I, *Sma*I, and *Bam*H1 cloning site; Summers and Invitrogen), and pBlue*Bac*III (*Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, for example, pAc700 (*Bam*H1 and *Kpn*I cloning site, in which the *Bam*H1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360 (*Bam*H1 cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with *Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and

*Hind*III cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen) can be used.

Mammalian vectors contemplated for use in the invention include, for example, vectors with inducible promoters, for example, the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, for example, pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current Protocols in Molecular Biology*, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, for example, pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, for example, pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive Rous Sarcoma Virus Long Terminal Repeat (RSV-LTR) promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive human cytomegalovirus (hCMV) immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection; Invitrogen), pcDNA3

(*Hind*III, *Kpn*I, *BamHI*, *BstXI*, *EcoR*I, *EcoR*V, *BstX*I [repeat], *Not*I, *Xho*I, *Xba*I, *Apa*I, cloning sites, G418, ampicillin selection, Invitrogen) and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman, 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and β-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and β-gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

A variety of methods may be used to confirm that the desired DNA sequence encoding TRAF2TR, TRAF2TD or TRAF2TR/2TD variants have been cloned into a vector. In general, one or more of the following approaches is used: (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of selection marker gene functions, (d) analyses with appropriate restriction endonucleases, and (e) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR to provide for detection of the amplified product. In the second approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted marker gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "selection marker" gene functions (*e.g.*, β-galactosidase activity, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In another example, if the nucleic acid encoding TRAF2TR is inserted within the "selection marker" gene sequence of the vector, recombinants containing the TRAF2TR insert can be identified by the absence of the selection marker gene function. In the fourth approach, recombinant expression vectors are identified by digestion with appropriate restriction enzymes. In the fifth approach, recombinant expression vectors can be identified by

assaying for the activity, biochemical, or immunological characteristics of the gene product expressed by the recombinant, provided that the expressed protein assumes a functionally active conformation.

Promoters

The nucleotide sequence coding for TRAF2TR or TRAF2TD or a TRAF2TR/2TD variant thereof can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding the polypeptides of the invention is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin. The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding TRAF2 and/or its flanking regions. Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control TRAF2TR gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature*

296:39-42); prokaryotic expression vectors for example, the β-lactamase promoter (Villa-Kamaroff, et al., 1978, *Proc. Natl. Acad. Sci. U.S.A.*, 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.*, 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; promoter elements from yeast or other fungi for example, the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Ornitz et al., 1986, Cold Spring Harbor *Symp. Quant. Biol.*, 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, *Cell* 38:647-658; Adames et al., 1985, *Nature* 318:533-538; Alexander et al., 1987, *Mol. Cell. Biol.*, 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, *Cell* 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, *Genes and Devel.* 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, *Mol. Cell. Biol.*, 5:1639-1648; Hammer et al., 1987, *Science* 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, *Genes and Devel.*, 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, *Nature* 315:338-340; Kollias et al., 1986, *Cell* 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, *Cell* 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, *Nature* 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, *Science* 234:1372-1378).

Introduction of Vectors into Host Cells

Vectors can be introduced into host cells by any suitable method, including, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, *J. Biol. Chem.* 267:963-967; Wu and Wu, 1988, *J. Biol. Chem.* 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990), so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facilitates purification for subsequent insertion into an appropriate expression cell line. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences from the yeast 2 μm plasmid.

Host Cell Systems

Potential host cell systems include but are not limited to mammalian host cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect host cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host cell system utilized, any one of a number of suitable transcription and translation elements may be used.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to

ensure the desired modification and processing of the foreign protein expressed. Expression in yeast can produce a biologically active product. Expression in eukaryotic cells can increase the likelihood of "native" folding. Moreover, expression in mammalian cells can provide a tool for reconstituting, or constituting, TRAF2TR-inhibiting activity. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent. Expression vectors of the invention can be used, as pointed out above, both to transfect cells for screening or biological testing of modulators of TRAF2TR activity.

A recombinant TRAF2TR, TRAF2TD or TRAF2TR/2TD variant of the invention may be expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

The cell into which the recombinant vector comprising the nucleic acid encoding TRAF2TR is introduced is cultured in an appropriate cell culture medium under conditions that provide for expression of TRAF2TR by the cell.

Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. Soluble forms of the protein can be obtained by collecting culture fluid, or solubilizing inclusion bodies, *e.g.*, by treatment with detergent, and if desired sonication or other mechanical processes, as described above. The solubilized or soluble protein can be isolated using various techniques, including polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, 2-dimensional gel electrophoresis, chromatography (*e.g.*, ion exchange, affinity, immunoaffinity, and sizing column chromatography), centrifugation, differential solubility, immunoprecipitation, or by any other standard technique for the purification of proteins.

As discussed above, a "vector" is any means for the transfer of a nucleic acid according to the invention into a host cell. Preferred vectors are viral vectors, for example, retroviruses, herpes viruses, adenoviruses, and adeno-associated viruses. Thus, a gene encoding a protein or polypeptide domain fragment of the present invention is introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both.

Use of Viral Vector Systems for
***ex vivo* and *in vivo* Treatment Methods**

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art [*see, e.g.*, Miller and Rosman, *BioTechniques* 7:980-990 (1992)]. Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors which are used within the scope of the present invention lack at least one region which is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), or be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques may be performed *in vitro* (on the isolated DNA) or *in situ*, using the techniques of genetic manipulation or by treatment with mutagenic agents. Preferably, the replication defective virus retains the sequences of its genome which are necessary for encapsulating the viral particles.

DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), vaccinia virus, and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not replication competent after introduction into a cell, and thus does not lead to a productive viral infection. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt et al., *Molec. Cell. Neurosci.* 2:320-330 (1991)], defective herpes virus vector lacking a glyco-protein L gene [Patent Publication RD 371005 A], or other defective herpes virus vectors [International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994]; an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. [*J. Clin. Invest.* 90:626-630 (1992); *see also* La Salle et al., *Science* 259:988-990 (1993)]; and a defective adeno-associated virus vector [Samulski et al., *J. Virol.* 61:3096-3101 (1987); Samulski et al., *J. Virol.* 63:3822-3828 (1989); Lebkowski et al., *Mol. Cell. Biol.* 8:3988-3996 (1988)].

Preferably, for *in vivo* administration, an appropriate immunosuppressive treatment is employed in conjunction with the viral vector, *e.g.*, adenovirus vector, to avoid immuno-deactivation of the viral vector and transfected cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon-g (IFN-g), or anti-CD4 antibody, can be administered to block humoral or cellular immune responses to the viral vectors [*see, e.g.*, Wilson, *Nature Medicine* (1995)]. In addition, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

Naturally, the invention contemplates delivery of a vector that will express a therapeutically effective amount of TRAF2TR for gene therapy applications. The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce and most preferably prevent an immune response resulting in a clinically significant manifestation of a disease linked to TNF α binding. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

Preferred Viral Vector Systems
Used in *ex vivo* and *in vivo* Treatment Methods

Certain viral vector systems are well developed in the art and are suited to the treatment methods of the present invention.

a. Adenovirus Vector Systems

In a preferred embodiment, the vector is an adenovirus vector. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard et al., *Virology* 75 (1990) 81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (e.g. Manhattan or A26/61 strain (ATCC VR-800), for example).

Preferably, the replication defective adenoviral vectors of the invention comprise the ITRs, an encapsidation sequence and the nucleic acid of interest. Still more preferably, at least the E1 region of the adenoviral vector is non-functional. The deletion in the E1 region preferably extends

from nucleotides 455 to 3329 in the sequence of the Ad5 adenovirus (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment). Other regions may also be modified, in particular the E3 region (WO95/02697), the E2 region (WO94/28938), the E4 region (WO94/28152, WO94/12649 and WO95/02697), or in any of the late genes L1-L5.

In a preferred embodiment, the adenoviral vector has a deletion in the E1 region (Ad 1.0). Examples of E1-deleted adenoviruses are disclosed in EP 185,573, the contents of which are incorporated herein by reference. In another preferred embodiment, the adenoviral vector has a deletion in the E1 and E4 regions (Ad 3.0). Examples of E1/E4-deleted adenoviruses are disclosed in WO95/02697 and WO96/22378, the contents of which are incorporated herein by reference. In still another preferred embodiment, the adenoviral vector has a deletion in the E1 region into which the E4 region and the nucleic acid sequence are inserted (see FR94 13355, the contents of which are incorporated herein by reference).

The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levrero et al., *Gene* 101 (1991) 195, EP 185 573; Graham, *EMBO J.* 3 (1984) 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid which carries, inter alia, the DNA sequence of interest. The homologous recombination is effected following cotransfection of the adenovirus and plasmid into an appropriate cell line. The cell line which is employed should preferably (i) be transformable by the said elements, and (ii) contain the sequences which are able to complement the part of the genome of the replication defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines which may be used are the human embryonic kidney cell line 293 (Graham et al., *J. Gen. Virol.* 36 (1977) 59) which contains the left-hand portion of the genome of an Ad5 adenovirus (12%) integrated into its genome, and cell lines which are able to complement the E1 and E4

functions, as described in applications WO94/26914 and WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

b. Adeno-Associated Virus Vector Systems

The adeno-associated viruses (AAV) are DNA viruses of relatively small size which can integrate, in a stable and site-specific manner, into the genome of the cells which they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterised. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions which carry the encapsulation functions: the left-hand part of the genome, which contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, which contains the cap gene encoding the capsid proteins of the virus.

The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (see WO 91/18088; WO 93/09239; US 4,797,368, US 5,139,941, EP 488 528). These publications describe various AAV-derived constructs in which the rep and/or cap genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the said gene of interest in vitro (into cultured cells) or in vivo, (directly into an organism). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsulation genes (rep and cap genes), into a cell line which is infected with a human helper virus (for example an adenovirus). The AAV

recombinants which are produced are then purified by standard techniques.

The invention also relates, therefore, to an AAV-derived recombinant virus whose genome encompasses a sequence encoding a nucleic acid encoding TRAF2TR or TRAF2TD flanked by the AAV ITRs. The invention also relates to a plasmid encompassing a sequence encoding a nucleic acid encoding TRAF2TR or TRAF2TD flanked by two ITRs from an AAV. Such a plasmid can be used as it is for transferring the nucleic acid sequence, with the plasmid, where appropriate, being incorporated into a liposomal vector (pseudo-virus).

c. Retrovirus Vector Systems

In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson et al., U.S. Patent No. 5,399,346; Mann et al., 1983, *Cell* 33:153; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., 1988, *J. Virol.* 62:1120; Temin et al., U.S. Patent No. 5,124,263; EP 453242, EP178220; Bernstein et al. *Genet. Eng.* 7 (1985) 235; McCormick, *BioTechnology* 3 (1985) 689; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty et al.; and Kuo et al., 1993, *Blood* 82:845. The retroviruses are integrating viruses which infect dividing cells. The retrovirus genome includes two LTRs, an encapsulation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the *gag*, *pol* and *env* genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Defective retroviral vectors are disclosed in WO95/02697.

In general, in order to construct recombinant retroviruses containing a nucleic acid sequence, a plasmid is

constructed which contains the LTRs, the encapsulation sequence and the coding sequence. This construct is used to transfect a packaging cell line, which cell line is able to supply in trans the retroviral functions which are deficient in the plasmid. In general, the packaging cell lines are thus able to express the gag, pol and env genes. Such packaging cell lines have been described in the prior art, in particular the cell line PA317 (US 4,861,719); the PsiCRIP cell line (WO90/02806) and the GP+envAm-12 cell line (WO89/07150). In addition, the recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsulation sequences which may include a part of the gag gene (Bender et al., *J. Virol.* 61 (1987) 1639). Recombinant retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

Retroviral vectors can be constructed to function as infections particles or to undergo a single round of transfection. In the former case, the virus is modified to retain all of its genes except for those responsible for oncogenic transformation properties, and to express the heterologous gene. Non-infectious viral vectors are prepared to destroy the viral packaging signal, but retain the structural genes required to package the co-introduced virus engineered to contain the heterologous gene and the packaging signals. Thus, the viral particles that are produced are not capable of producing additional virus. Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Non-Viral Systems Used in
<u>*ex vivo* and *in vivo* Treatment Methods</u>

Certain non-viral systems have been used in the art and can facilitate introduction of DNA encoding the polypeptides of the present invention to desired target cells.

a. Lipofection Delivery Systems

A vector can be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker [Felgner, et. al., *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417 (1987); *see* Mackey, et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:8027-8031 (1988); Ulmer et al., *Science* 259:1745-1748 (1993)]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner and Ringold, *Science* 337:387-388 (1989)]. Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Patent No. 5,459,127. The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, for example, pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting [*see* Mackey, et. al., *supra*]. Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins for example, antibodies, or non-peptide molecules could be coupled to liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, for example, a cationic oligopeptide (*e.g.*, International Patent Publication WO95/21931), peptides derived from DNA binding proteins (*e.g.*, International Patent Publication WO96/25508), or a cationic polymer (*e.g.*, International Patent Publication WO95/21931).

b. Naked DNA Delivery Systems

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid (see U.S. Patents 5,693,622, 5,589,466 and 5,580,859). Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter [*see, e.g.*, Wu et al., *J. Biol. Chem.* 267:963-967 (1992); Wu and Wu, *J. Biol. Chem.* 263:14621-14624 (1988); Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams et al., *Proc. Natl. Acad. Sci. USA* 88:2726-2730 (1991)]. Receptor-mediated DNA delivery approaches can also be used [Curiel et al., *Hum. Gene Ther.* 3:147-154 (1992); Wu and Wu, *J. Biol. Chem.* 262:4429-4432 (1987)].

Methods to Identify
<u>Therapeutically Useful Variants of TRAF2TR</u>

There are a variety of methods which may be used to determine whether a TNF α regulated pathway is involved in a disease state and to determine the effect of the polypeptides of the present invention on these pathways. For example, to study the role of TRAF2TR in NFκB regulation, electrophoretic mobility shift assay (EMSA) analysis was performed using NFκB UAS as a probe (Figure 7). Nuclear extracts from cells overexpressing FL TRAF (lanes 3 and 4) show TNF α induced shifts significantly stronger in comparison to control (lanes 1 and 2). TRAF2TR overexpression blocks formation of NFκB and, as a result, no shift has been detected in TNF α stimulated cells (lanes 5 and 6). These results suggested strong inhibition of NFκB formation as no shift band appeared in TNF α stimulated cells. Increased amount of NF κB binding activity is present in cells overexpressing full-length TRAF2 after stimulation with TNF α (lane 4).

Experiments of the type discussed hereinabove can be utilized to determine whether a given pathway implicated in a disease state might be treated using the compositions of the

present invention. In general, the experiments described above can be performed substituting TRAF2TR or TRAF2TD with an isolated or prepared variant of TRAF2 to determine if the variant has the ability to inhibit TNF α regulated pathways.

Disease States Related to TNF α Regulated Pathways

As discussed above, the present invention relates to the use of TRAF2TR and TRAF2TD and variants thereof to inhibit TNF α regulated pathways. In particular, the present invention relates to using the aforementioned to effectively block TNF α induced activation of several transcriptional factors, including NFκB and AP-1. TRAF2TR and TRAF2TD and variants thereof are useful in inhibiting TNF α signal transduction pathways in pathologies which involve overproduction of TNF α and hyperactivation of NFκB dependent genes. A variety of diseases appear to involve TNF α regulated pathways and the pathological basis for these diseases may involve overproduction of TNF α or hyperactivation of NFκB dependent genes. These diseases can be treated using the TRAF2TR and TRAF2TD proteins and their variants.

Given the evidence that inflammatory processes contribute heavily to the pathology of all the major cardiovascular disease states and given that elevated TNF α levels are associated with these inflammatory processes, it is believed that a variety of major cardiovascular disease states can be treated using the compositions and methods of the present invention. These diseases include, but are not limited to, cardiovascular disease states, including cardiac ischemia-reperfusion injury following myocardial infarction, coronary artery bypass surgery, cardiac transplantation or ischemia-reperfusion injury in the CNS following stroke; the progression and rupture of advanced coronary atherosclerotic plaques; the development and progression of congestive heart failure; and endothelial cell injury following balloon angioplasty. In addition, the present invention can be used to prevent apoptotic cell death of myocardial cells during heart failure or infarction and to avoid myocyte apoptosis.

By blocking TNF α receptor signaling, a gene therapy approach using TRAF2TR or TRAF2TD or a variant thereof can be used to treat these diseases. The use of TRAF2TD is preferred, given its highly effective inhibition of TNF α regulated pathways.

Similarly, by blocking TNF α receptor signaling, gene therapy with TRAF2TR of TRAF2TD or a variant thereof can be used to treat other disease states where TNF α is involved in the pathogenesis. These disease states include, but are not limited to, Crohn's disease, psoriasis, rheumatoid arthritis, graft versus host disease, inflammatory bowel disease, non-insulin dependent diabetes and neurodegenerative diseases (e.g., Parkinson's disease).

Additionally, TRAF2TD can be used in various assays to study the mechanisms of TRAF2-dependent signal transduction pathways.

__Therapeutic Compositions and Dosages__

In use, any vector, viral or non-viral, of the invention is preferably introduced *in vivo* in a pharmaceutically acceptable vehicle or carrier. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce a significant allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, for example, water and oils, including those of petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and

aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

The present invention provides methods of treatment which comprise the administration to a human or other animal of an effective amount of a composition of the invention.

Effective amounts may vary, depending on the age, type and severity of the condition to be treated, body weight, desired duration of treatment, method of administration, and other parameters. Effective amounts are determined by a physician or other qualified medical professional.

It is believed that polypeptides according to the invention will be used most widely in doses of about 0.01 mg/kg to about 100 mg/kg of body weight per day. Preferred doses are about 0.1 mg/kg to about 50 mg/kg, with doses of about 1 mg/kg to about 10 mg/kg of body weight per day being more preferred.

Recombinant viruses according to the invention are generally formulated and administered in the form of doses of between about 10^4 and about 10^{14} pfu. In the case of AAVs and adenoviruses, doses of from about 10^6 to about 10^{11} pfu are preferably used. The term "pfu" ("plaque-forming unit") corresponds to the infective power of a suspension of virions and is determined by infecting an appropriate cell culture and measuring the number of plaques formed. The techniques for determining the pfu titre of a viral solution are well documented in the prior art.

The following examples are illustrative of the present invention.

EXAMPLES

EXAMPLE 1 - Isolation of cDNA Encoding TRAF2TR

The TRAF2 splice variant, TRAF2TR, was isolated during RT PCR cloning (Current Protocols in Molecular Biology, 1996) of full length TRAF2 using mRNA from the human Osteosarcoma cell line (OSA1). While using primers to produce full length TRAF2 cDNA, a smaller PCR product was observed. The fragment was excised and cloned independently. The 5' primer used for the RT PCR was ATG GCT GCA GCT AGC GTG ACC and the 3' primer was TTA TAG CCC TGT CAG GTC CAC.

Upon sequencing of this smaller clone (TRAF2TR), an in-frame deletion of the codons encoding amino acid residues 123 to 201 were identified. Figures 4a and 4b compare the nucleic and amino acid sequences of full length TRAF2 and TRAF2TR.

A variety of tissues in the body have been identified as sources of TRAF2TR mRNA and may be used to isolate the TRAF2TR mRNA using the protocol described above. To determine the tissue distribution of TRAF2TR, RT-PCR was performed using a pair of primers outside of the spliced region. The primer on the 5' side of the deletion was: GGT GGA GAG CCT GCC GGC CG and the primer on the 3' side of the deletion was: GGC AGC CGA TGG CGT GGA ATC TG, and cDNA was generated using an oligo-dT primer from total RNA from different tissues. The cDNAs were separated by agar electrophoresis and transferred to nitrocellulose. Hybridization was performed using a specific probe from the TRAF2 sequence adjacent to the 5' end of the spliced region (5' - GAT GCA CCT GGA AGG GGA CCC TGA AAT - 3'). This probe recognizes both non-spliced and spliced variants of TRAF2. The expected size for the non-spliced variant (TRAF2FL) is 373 bp and for the spliced variant (TRAF2TR), 136 bp. Referring now to Figure 5, Lanes: 1-control TRAF2-FL cDNA; 2-control TRAF2 spliced variant cDNA; 3-Jurkat; 4-HeLa cell line; 5-thymus; 6-placenta; 7-thymus; 8-spleen; 9-ovary.

Western blot analysis of lysates from various cell sources does not unequivocally detect the presence of the truncated TRAF2 variant at the level of expressed protein. It appears that the high level expression and production of the protein are limited developmentally, temporally, or controlled by an undefined mechanism, in a cell type dependent manner (e.g., B cell maturation in Germinal centers.).

The deletion in the splice variant TRAF2TR retains an open reading frame and the 5' splice boundary matches canonical splice donor sequence. The deletion removes amino acid residues 123-201 of WT TRAF2, which encompasses the C-terminal portion of zinc finger domain 1 and all of zinc fingers 2 and 3 as well as the N-terminal residues of zinc finger 4 (Figure 1). This deletion more than likely disrupts the function of the zinc finger region, and is similar to the deletion created by Takeuchi et al., J. Biol. Chem. 271(33), 19935-19942 (1996) which they report exhibits a dominant negative effect on TNF α induced NFKB activation.

EXAMPLE 2 - Preparation of TRAF2TD

It is known that deletion of N-amino terminal 87 amino acids (representing the ring fingers domain, see Figure 1) of TRAF2 creates a protein which acts as a dominant inhibitor (dominant negative) of TNF α dependent NFκB activation (Takeuchi et al., *J. Biol. Chem.* 271(33), 19935-19942 (1996)). In order to determine if an N-terminal deletion would affect the activity of TRAF2TR, a construct representing TRAF2TR with a deletion of 87 amino acids from the N-terminus of the protein (residues 1 to 87) was prepared. To prepare this variant of TRAF2TR, TRAF2TR cDNA was used as a template for PCR using a 5' primer encompassing nucleotides 262 to 280 of the TRAF2 full length coding sequence (ATGAGTTCGGCCTTCCCAGAT wherein the ATG codon was included to create a translation initiation site; the 3' primer was TTA TAG CCC TGT CAG GTC CAC. The resulting construct begins at amino acid 88 of full length TRAF2 and contains the 123 to 201 amino acid deletion of TRAF2TR,

providing a "double deletion" construct. The construct was verified by DNA sequencing and cloned into a mammalian expression vector (pcDNA3, Invitrogen).

EXAMPLE 3 - Transfection of HeLa Cells with TRAF2TR

To determine the effect of TRAF2TR on NFκB activation, truncated as well as the full length (FL) TRAF2 were constructed with N-Myc affinity tags in a mammalian expression vector (pcDNA3, INVITROGEN). To prepare N-myc fusion constructs, a 5' PCR primer was synthesized containing the sequence of the Myc tag with a starting methionine (MetGluGlnLysLeuIleSerGluGluAspLeuAsn) followed by the first 20 nucleotides of the TRAF2 cDNA: 5' - ATG GAG CAG AAA TTG ATT TCC GAG GAA GAT CTG AAC ATG GCT GCA GCT AGC GTG AC - 3'. The 3' PCR primer sequence was: 5' - TTAGAGCCCTGTCAGGTCCACAA - 3'. The PCR product was purified and cloned into the pcDNA3 vector using standard techniques.

HeLa cells were transfected with pcDNA3-myc TRAF2 constructs using LipoFectamine (BRL, Gibco) reagent using the protocol provided by the reagent supplier. In brief, 4 ml of LipoFectamine were mixed with 1 mg of the DNA in 1 ml of Serum free DMEM (BRL) media and 3 x 10^5 cells in 60 mm Petri dish were incubated with that mixture overnight at 37°C at 5% CO_2 incubator. Twenty-four hours after transfection, cells were washed with phosphate buffered saline and lysed in 200 ml of an SDS electrophoresis sample buffer (SIGMA). Ten ml of the lysate was separated by electrophoresis and Western blotted to a nitrocellulose membrane. Immunostaining and ECL (AMERSHAM) detection was performed according to the recommendations of the antibody supplier.

Anti-Myc antibodies (BABCO, Berkeley) detected proteins of the expected size in HeLa cells transfected with pcDNA-TRAF2FL and pcDNA-TRAF2TR (Fig. 6).

The results, shown in Figure 6, show the immunodetection of Myc-fused TRAF2FL and TRAF2TR in transfected HeLa cells. HeLa cells were transfected with expression constructs of

TRAF-FL and TRAF-TR using lipofectamine (Gibco BFL) and after 24 hours cells were lysed in SDS loading buffer. Myc-fusion proteins were detected using anti-myc Ab and ECL detection system. Lanes: 1-pcDNA3 vector; 2-myc-TRAF2FL; 3-myc-TRAF2TR.

EXAMPLE 4 - NFκB Reporter System

In order to determine the effect of TRAF2TR, TRAF2TD or variants of these polypeptides on NFκB regulated gene expression, an NFκB reporter system can be used, such as the system utilized and described in Takeuchi et al., *J. Biol. Chem.*, 271(33), 19935-42 (1996). An NFκB reporter activation system may be utilized in conjunction with appropriate cells, such as 293 cells, COS7 cells or HeLa cells. The cells would be transfected with different TRAF2 constructs, i.e., full length TRAF2, TRAF2TR and TRAF2TD, using the lipofectamine protocol discussed in Example 3. The effect of the different TRAF2 fragments on activation of a cotransfected NFκB reporter can then be compared to identify the most effective inhibitor species. As an example, TRAF2 constructs comprising full length TRAF2, TRAF2TR and TRAF2TD, as well as variants of TRAF2TR and TRAF2TD, can be transfected into 293 cells and the level of NFκB reporter activity monitored in the presence or absence of TNFα. The full-length TRAF2 would be expected to activate the cotransfected NFκB reporter while the other TRAF2 constructs would be expected to block TNF α mediated activation of the NFκB reporter to varying degrees.

EXAMPLE 5 - *Ex Vivo* Treatment Methods

Methods of *ex vivo* gene therapy are known in the art and generally involve four stages. In the first stage, cells of a given type are collected from the patient to be treated. In the second stage, the desired gene is transfected into the isolated cells. In the third stage, those cells which have taken up the desired gene are selected and grown. In the fourth stage, the cells are either infused or transplanted back into the patient where they express the desired gene and treat the disease.

In the first stage of an *ex vivo* treatment method utilizing the present invention, cells are obtained from the patient. The choice of cell is based on a number of factors, primarily the specific disease being treated. Blocking activation in these target cells would inhibit expression of pro-inflammatory cytokines and other proteins involved in the inflammatory processes linked to manifestation of a cardiovascular disease state.

In the second stage, the TRAF2TR or TRAF2TD or variant cDNA is cloned into an appropriate mammalian expression vector. For transfection protocols involving lipofection, an expression vector, for example, pcDNA3 can be used. In a preferred embodiment, TRAF2TD, given its enhanced ability to inhibit TNF α binding effects, is cloned into pcDNA3 or another suitable mammalian expression vector. The promoter utilized in the expression vector is chosen based on the type of cells being transfected and the desired method for regulating the level of expression. An appropriate promoter can be selected from among the promoters discussed *supra*. For gene therapy of heart diseases, a promoter, for example, 2MHC (see Palermo et al., *Circ. Res.*, 78(3), 504-9 (1996)), MLC2 (see Sani, *Nature*, 314:283-286 (1985)), CARP (see Jeyaseelan et al., *J. Biol. Chem.*, 272(36), 22800-8 (1997)), is inserted upstream of the TRAF2TD cDNA. The TRAF2TD cDNA containing expression vector is then used in a liposome-mediated transfection utilizing lipofectamine (BRL, Gibco) reagent using the supplied protocol. For transfection protocols using viral transduction, the second stage of the *ex vivo* treatment protocol utilizes a recombinant adenovirus vector system. In this protocol, the TRAF2TD cDNA is cloned into an adenovirus expression vector, for example, adeno-quest pQBI-AdBN/NB (QUANTUM Biotechnologies Inc.). The adenoviral transfer vector now containing the TRAF2TD cDNA is then co-transfected with adenovirus viral DNA into 293 cells. Following plaque purification and positive clone selection, recombinant adenovirus containing the TRAF2TD cDNA is amplified and then purified using conventional CsCl step gradient purification, followed by dialysis using an

appropriate buffer, for example, phosphate buffered saline. The recombinant adenovirus is then used to transfect the target cells *ex vivo*.

Recombinant viruses according to the invention are formulated and administered in the form of doses of between about 10^4 and about 10^{14} pfu. In the case of AAVs and adenoviruses, doses of from about 10^6 to about 10^{11} pfu are preferably used. The term "pfu" ("plaque-forming unit") corresponds to the infective power of a suspension of virions and is determined by infecting an appropriate cell culture and measuring the number of plaques formed. The techniques for determining the pfu titre of a viral solution are well documented in the prior art.

In the third stage, the transfected cells obtained by either lipofection or recombinant adenovirus infection are grown up in culture, selecting for those cells which have been transfected. Selection can be done in a variety of ways, including using a drug marker that provides for survival and growth of only those cells which have taken up the expression vector.

In the fourth stage, the transfected cells are infused or transplanted directly into the patient, either near the tissue to be treated or at a location that allows the TRAF2TD cDNA product to be released into the circulation so as to interact with the cells subject to activation by TNF α binding. Delivery means include, but are not limited to, direct injection, or delivery by catheter, infusion pump or stent.

EXAMPLE 6 - *In Vivo* Treatment Methods

Methods of *in vivo* treatment can utilize a variety of different viral vectors, including adenovirus vectors, adeno-associated virus vectors, and retrovirus vectors. In a preferred *in vivo* treatment method of the present invention, an adenovirus system is used to introduce the TRAF2TR or TRAF2TD cDNA into host cells. Given the relatively greater

ability of the TRAF2TD cDNA to inhibit TNF α binding activation, it is preferable to use the TRAF2TD cDNA in the adenovirus expression vector. In this method, the TRAF2TD cDNA is cloned into an adenovirus transfer vector, for example, the adeno-Quest pQBI-AdBN/NB (QUANTUM Biotechnologies Inc.) or another adenovirus vector from those described *supra*. The promoter utilized in the expression vector is chosen based on the type of cells being transfected and the desired method for regulating the level of expression. An appropriate promoter can be selected from among the promoters discussed *supra*.

The adenoviral transfer vector containing the desired promoter and the TRAF2TD cDNA would be then co-transfected with adenovirus viral DNA into 293 cells. Following plaque purification and positive clone selection, recombinant adenovirus containing the TRAF2TD cDNA is amplified and then purified using conventional CsCl step gradient purification, followed by dialysis using an appropriate buffer, for example, phosphate buffered saline.

Prior to transfecting cells *in vivo*, viral particle titer is determined and experiments *in vitro* are performed to determine the level of protein expression and the tissue culture infectious dose (TCID50). Recombinant viruses according to the invention are formulated and administered in the form of doses of between about 10^4 and about 10^{14} pfu. In the case of AAVs and adenoviruses, doses of from about 10^6 to about 10^{11} pfu are preferably used. The term "pfu" ("plaque-forming unit") corresponds to the infective power of a suspension of virions and is determined by infecting an appropriate cell culture and measuring the number of plaques formed. The techniques for determining the pfu titre of a viral solution are well documented in the prior art.

The recombinant adenovirus would then be used to infect the patient at a dose of between about 10^6 to about 10^{11} pfu. The recombinant adenovirus may be introduced by inhalation,

by infusion, by surgical implantation, by direct injection or delivery by catheter, infusion pump or stent.

We claim:

1. A DNA sequence encoding TRAF2TR comprising the sequence as shown in Figure 2a.

2. A DNA sequence encoding TRAF2TD comprising the sequence as shown in Figure 3a.

3. The DNA of Claim 1 wherein said DNA is a cDNA.

4. The DNA of Claim 2 wherein said DNA is a cDNA.

5. An isolated and purified TRAF2TR polypeptide which is capable of inhibiting TNF α-regulated pathways and comprising an amino acid sequence as shown in Figure 2b.

6. A TRAF2TD polypeptide which is capable of inhibiting TNF α-regulated pathways and comprising an amino acid sequence as shown in Figure 3b.

7. A method of inhibiting TNF α-regulated pathways in a patient comprising introducing into the body of said patient a composition capable of inhibiting TNF α regulated pathways.

8. The method of Claim 7 wherein said composition is an expression vector capable of expressing TRAF2TR polypeptide.

9. The method of Claim 7 wherein said composition is an expression vector capable of expressing TRAF2TD polypeptide.

10. The method of Claim 7 wherein said composition comprises a TRAF2TR polypeptide and a pharmaceutically acceptable carrier.

11. The method of Claim 7 wherein said composition comprises a TRAF2TD polypeptide and a pharmaceutically acceptable carrier.

12. A method of inhibiting diseases involving overproduction of TNF α comprising introducing into the body of a patient a composition capable of inhibiting TNF α regulated pathways.

13. The method of Claim 12 wherein said composition comprises an expression vector capable of expressing TRAF2TR.

14. The method of Claim 12 wherein said composition comprises an expression vector capable of expressing TRAF2TD.

15. The method of Claim 12 wherein said composition is a TRAF2TR polypeptide and a pharmaceutically acceptable carrier.

16. The method of Claim 12 wherein said composition is a TRAF2TD polypeptide and a pharmaceutically acceptable carrier.

17. A method of inhibiting TNF α pathologies involving hyperactivation of nuclear factor $\kappa\beta$ (NFKB) dependent genes comprising introducing into a patient a composition capable of inhibiting TNF α regulated pathways.

18. The method of Claim 17 wherein said composition is an expression vector capable of expressing TRAF2TR.

19. The method of Claim 17 wherein said composition is an expression vector capable of expressing TRAF2TD.

20. The method of Claim 17 wherein said composition is a TRAF2TR polypeptide and a pharmaceutically acceptable carrier.

21. The method of Claim 17 wherein said composition is a TRAF2TD polypeptide and a pharmaceutically acceptable carrier.

22. A method of inhibiting inflammatory processes involving TNF α comprising introducing into a patient's body a composition capable of inhibiting TNF α regulated pathways.

23. The method of Claim 22 wherein said composition is an expression vector capable of expressing TRAF2TR.

24. The method of Claim 22 wherein said composition is an expression vector capable of expressing TRAF2TD.

25. The method of Claim 22 wherein said composition is a TRAF2TR polypeptide and a pharmaceutically acceptable carrier.

26. The method of Claim 22 wherein said composition is a TRAF2TD polypeptide and a pharmaceutically acceptable carrier.

27. The method of Claim 22 wherein said inflammatory processes involving TNF α is selected from the group consisting of Crohn's disease, psoriasis, rheumatoid arthritis, graft versus host disease, inflammatory bowel disease, non-insulin dependent diabetes and neurogenerative diseases.

28. The method of Claim 22 wherein said inflammatory process involving TNF α is a cardiovascular disease.

29. The method of Claim 28 wherein said cardiovascular disease is selected from the group consisting of (a) cardiac ischemia-reperfusion injury following myocardial infarction, coronary artery bypass surgery, cardiac transplantation or ischemia-reperfusion injury in the CNS following stroke; (b) the progression and rupture of advanced coronary atherosclerotic plaques; (c) the development and progression

of congestive heart failure; (d) endothelial cell injury following balloon angioplasty; and (e) apoptotic cell death of myocardial cells.

30. A DNA sequence encoding a TRAF2TR/2TD variant.

31. The DNA sequence of Claim 30 wherein said DNA sequence includes conservative amino acid substitutions.

32. A TRAF2TR/2TD variant polypeptide which is capable of inhibiting TNFα-regulated pathways.

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(54) Title: VARIANTS OF TRAF2 WHICH ACT AS AN INHIBITOR OF TNF-ALPHA (TNF α) SIGNALING PATHWAY

(57) Abstract: The present invention relates to variants of TRAF2 which demonstrate the ability to inhibit the TNF α signaling pathway. In particular, applicants have isolated a splice variant of TRAF2 referred to hereinafter as "TRAF2 truncated" or "TRAF2TR" and a TRAF2 expression construct with enhanced dominant negative properties, hereafter referred to as "TRAF2 truncated-deleted" or "TRAF2TD". Both TRAF2TR and TRAF2TD have the ability to inhibit the TNF α signaling pathway and in TRAF2TD, this ability is greatly enhanced, greatly reducing the response to TNF α binding.

Structure of TRAF2 and the alternatively spliced variant, TRAF2-TR.

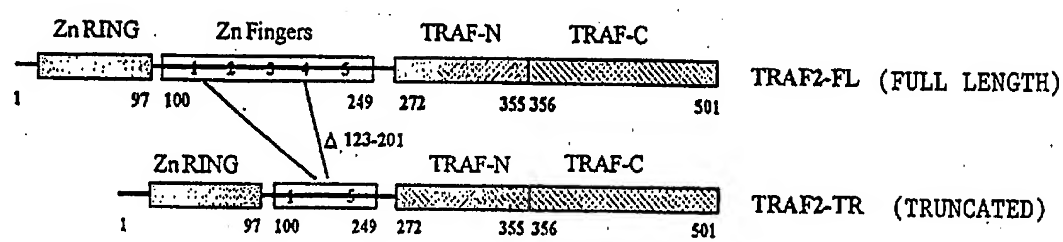

FIGURE 1

1 ATGGCTGCAGCTAGCGTGACCCCCCCTGGCTCCCTGGAGTTGCTACAGCCCGGCTTCTCCAAGACCCTCCTGGGGACCAA
GCTGGAAGCCAAGTACCTGTGCTCCGCCTGCAGAAACGTCCTCCGCAGGCCCTTCCAGGCGCAGTGTGGCCACCGGTACT
GCTCCTTCTGCCTGGCCAGCATCCTCAGCTCTGGGCCTCAGAACTGTGCTGCCTGTGTTCACGAGGGCATATATGAAGAA
GGCATTTCTATTTTAGAAAGCAGTTCGGCCTTCCCAGATAATGCTGCCCGCAGGGAGGTGGAGAGCCTGCCGGCCGTCTG
TCCCAGTGATGGATGCACCTGGAAGGGGACCCTGAAAGAATACGAGTTTCAGGACCACGTCAAGACTTGTGGCAAGTGTC
GAGTCCCTTGCAGATTCCACGCCATCGGCTGCCTCGAGACGGTAGAGGGTGAGAAACAGCAGGAGCACGAGGTGCAGTGG
CTGCGGGAGCACCTGGCCATGCTACTGAGCTCGGTGCTGGAGGCAAAGCCCCTCTTGGGAGACCAGAGCCACGCGGGGTC
AGAGCTCCTGCAGAGGTGCGAGAGCCTGGAGAAGAAGACGGCCACTTTTGAGAACATTGTCTGCGTCCTGAACCGGGAGG
TGGAGAGGGTGGCCATGACTGCCGAGGCCTGCAGCCGGCAGCACCGGCTGGACCAAGACAAGATTGAAGCCCTGAGTAGC
AAGGTGCAGCAGCTGGAGAGGAGCATTGGCCTCAAGGACCTGGCGATGGCTGACTTGGAGCAGAAGGTCTTGGAGATGGA
GGCATCCACCTACGATGGGGTCTTCATCTGGAAGATCTCAGACTTcgCCAGGAAGCtCCAGGAAGCTGTGGCTGGCCGCA
TACCCGCCATCTTCTCCCCAGCCTTCTACACCAGCAGGTACGGCTACAAGATGTGTCTGCGTATCTACCTGAACGGCGAC
GGCACCGGGCGAGGAACACACCTGTCCCTCTTCTTTGTGGTGATGAAGGGCCCGAATGACGCCCTGCTGCGGTGGCCCTT
CAACCAGAAGGTGACCTTAATGCTGCTCGACCAGAATAACCGGGAGCACGTGATTGACGCCTTCAGGCCCGACGTGACTT
CATCCTCTTTTCAGAGGCCAGTCAACGACATGAACATCGCAAGCGGCTGCCCCCTCTTCTGCCCCGTCTCCAAGATGGAG
GCAAAGAATTCCTACGTGCGGGACGATGCCATCTTCATCAAGGCCATTGTGGACCTGACAGGGCTCTAA 1269

FIGURE 2a

1 MAAASVTPPGSLELLQPGFSKTLLGTKLEAKYLCSACRNVLRRPFQAQCGHRYCSFCLASILSSGPQNCAACVHEGIYEE
GISILESSSAFPDNAARREVESLPAVCPSDGCTWKGTLKEYEFQDHVKTCGKCRVPCRFHAIGCLETVEGEKQQEHEVQW
LREHLAMLLSSVLEAKPLLGDQSHAGSELLQRCESLEKKTATFENIVCVLNREVERVAMTAEACSRQHRLDQDKIEALSS
KVQQLERSIGLKDLAMADLEQKVLEMEASTYDGVFIWKISDFARKLQEAVAGRIPAIFSPAFYTSRYGYKMCLRIYLNGD
GTGRGTHLSLFFVVMKGPNDALLRWPFNQKVTLMLLDQNNREHVIDAFRPDVTSSSFQRPVNDMNIASGCPLFCPVSKME
AKNSYVRDDAIFIKAIVDLTGL 422

FIGURE 2b

ATGAGTTCGGCCTTCCCAGATAATGCTGCCCGCAGGGAGGTGGAGAGCCTGCCGGCCGTCTG
TCCCAGTGATGGATGCACCTGGAAGGGGACCCTGAAAGAATACGAGTTTCAGGACCACGTCAAGACTTGTGGCAAGTGTC
GAGTCCCTTGCAGATTCCACGCCATCGGCTGCCTCGAGACGGTAGAGGGTGAGAAACAGCAGGAGCACGAGGTGCAGTGG
CTGCGGGAGCACCTGGCCATGCTACTGAGCTCGGTGCTGGAGGCAAAGCCCCTCTTGGGAGACCAGAGCCACGCGGGGTC
AGAGCTCCTGCAGAGGTGCGAGAGCCTGGAGAAGAAGACGGCCACTTTTGAGAACATTGTCTGCGTCCTGAACCGGGAGG
TGGAGAGGGTGGCCATGACTGCCGAGGCCTGCAGCCGGCAGCACCGGCTGGACCAAGACAAGATTGAAGCCCTGAGTAGC
AAGGTGCAGCAGCTGGAGAGGAGCATTGGCCTCAAGGACCTGGCGATGGCTGACTTGGAGCAGAAGGTCTTGGAGATGGA
GGCATCCACCTACGATGGGGTCTTCATCTGGAAGATCTCAGACTTcgCCAGGAAGCtCCAGGAAGCTGTGGCTGGCCGCA
TACCCGCCATCTTCTCCCCAGCCTTCTACACCAGCAGGTACGGCTACAAGATGTGTCTGCGTATCTACCTGAACGGCGAC
GGCACCGGGCGAGGAACACACCTGTCCCTCTTCTTTGTGGTGATGAAGGGCCCGAATGACGCCCTGCTGCGGTGGCCCTT
CAACCAGAAGGTGACCTTAATGCTGCTCGACCAGAATAACCGGGAGCACGTGATTGACGCCTTCAGGCCCGACGTGACTT
CATCCTCTTTTCAGAGGCCAGTCAACGACATGAACATCGCAAGCGGCTGCCCCCTCTTCTGCCCCGTCTCCAAGATGGAG
GCAAAGAATTCCTACGTGCGGGACGATGCCATCTTCATCAAGGCCATTGTGGACCTGACAGGGCTCTAA

FIGURE 3a

MSSAFPDNAARREVESLPAVCPSDGCTWKGTLKEYEFQDHVKTCGKCRVPCRFHAIGCLETVEGEKQQEHEVQW
LREHLAMLLSSVLEAKPLLGDQSHAGSELLQRCESLEKKTATFENIVCVLNREVERVAMTAEACSRQHRLDQDKIEALSS
KVQQLERSIGLKDLAMADLEQKVLEMEASTYDGVFIWKISDFARKLQEAVAGRIPAIFSPAFYTSRYGYKMCLRIYLNGD
GTGRGTHLSLFFVVMKGPNDALLRWPFNQKVTLMLLDQNNREHVIDAFRPDVTSSSFQRPVNDMNIASGCPLFCPVSKME
AKNSYVRDDAIFIKAIVDLTGL

FIGURE 3b

FIGURE 4a

```
           1                                                      50
TRAF2-TR   GAATTCCGGC GCGCTGCGAC CGTTGGGGCT TTGTTCGCGG GGGTCACAGC
TRAF2-FL   GAATTCCGGC GCGCTGCGAC CGTTGGGGCT TTGTTCGCGG GGGTCACAGC

           51                                                    100
TRAF2-TR   TCTCATGGCT GCAGCTAGCG TGACCCCCCC TGGCTCCCTG GAGTTGCTAC
TRAF2-FL   TCTCATGGCT GCAGCTAGCG TGACCCCCCC TGGCTCCCTG GAGTTGCTAC

           101                                                   150
TRAF2-TR   AGCCCGGCTT CTCCAAGACC CTCCTGGGGA CCAAGCTGGA AGCCAAGTAC
TRAF2-FL   AGCCCGGCTT CTCCAAGACC CTCCTGGGGA CCAAGCTGGA AGCCAAGTAC

           151                                                   200
TRAF2-TR   CTGTGCTCCG CCTGCAGAAA CGTCCTCCGC AGGCCCTTCC AGGCGCAGTG
TRAF2-FL   CTGTGCTCCG CCTGCAGAAA CGTCCTCCGC AGGCCCTTCC AGGCGCAGTG

           201                                                   250
TRAF2-TR   TGGCCACCGG TACTGCTCCT TCTGCCTGGC CAGCATCCTC AGCTCTGGGC
TRAF2-FL   TGGCCACCGG TACTGCTCCT TCTGCCTGGC CAGCATCCTC AGCTCTGGGC

           251                                                   300
TRAF2-TR   CTCAGAACTG TGCTGCCTGT GTTCACGAGG GCATATATGA AGAAGGCATT
TRAF2-FL   CTCAGAACTG TGCTGCCTGT GTTCACGAGG GCATATATGA AGAAGGCATT

           301                                                   350
TRAF2-TR   TCTATTTTAG AAAGCAGTTC GGCCTTCCCA GATAATGCTG CCCGCAGGGA
TRAF2-FL   TCTATTTTAG AAAGCAGTTC GGCCTTCCCA GATAATGCTG CCCGCAGGGA

           351                                                   400
TRAF2-TR   GGTGGAGAGC CTGCCGGCCG TCTGTCCCAG TGATGGATGC ACCTGGAAGG
TRAF2-FL   GGTGGAGAGC CTGCCGGCCG TCTGTCCCAG TGATGGATGC ACCTGGAAGG

           401                                                   450
TRAF2-TR   GGACCCTGAA AGAATACGAG ---------- ---------- ----------
TRAF2-FL   GGACCCTGAA AGAATACGAG AGCTGCCACG AAGGCCGCTG CCCGCTCATG

           451                                                   500
TRAF2-TR   ---------- ---------- ---------- ---------- ----------
TRAF2-FL   CTGACCGAAT GTCCCGCGTG TAAAGGCCTG GTCCGCCTTG GTGAAAAGGA

           501                                                   550
TRAF2-TR   ---------- ---------- ---------- ---------- ----------
TRAF2-FL   GCGCCACCTG GAGCACGAGT GCCCGGAGAG AAGCCTGAGC TGCCGGCATT
```

FIGURE 4a (continued)

```
          551                                                   600
TRAF2-TR  ---------- ---------- ---------- ---------- ----------
TRAF2-FL  GCCGGGCACC CTGCTGCGGA GCAGACGTGA AGGCGCACCA CGAGGTCTGC

          601                                                   650
TRAF2-TR  ---------- ---------- ---------- ---------- ----------
TRAF2-FL  CCCAAGTTCC CCTTAACTTG TGACGGCTGC GGCAAGAAGA AGATCCCCCG

          651                                                   700
TRAF2-TR  -------TTT CAGGACCACG TCAAGACTTG TGGCAAGTGT CGAGTCCCTT
TRAF2-FL  GGAGAAGTTT CAGGACCACG TCAAGACTTG TGGCAAGTGT CGAGTCCCTT

          701                                                   750
TRAF2-TR  GCAGATTCCA CGCCATCGGC TGCCTCGAGA CGGTAGAGGG TGAGAAACAG
TRAF2-FL  GCAGATTCCA CGCCATCGGC TGCCTCGAGA CGGTAGAGGG TGAGAAACAG

          751                                                   800
TRAF2-TR  CAGGAGCACG AGGTGCAGTG GCTGCGGGAG CACCTGGCCA TGCTACTGAG
TRAF2-FL  CAGGAGCACG AGGTGCAGTG GCTGCGGGAG CACCTGGCCA TGCTACTGAG

          801                                                   850
TRAF2-TR  CTCGGTGCTG GAGGCAAAGC CCCTCTTGGG AGACCAGAGC CACGCGGGGT
TRAF2-FL  CTCGGTGCTG GAGGCAAAGC CCCTCTTGGG AGACCAGAGC CACGCGGGGT

          851                                                   900
TRAF2-TR  CAGAGCTCCT GCAGAGGTGC GAGAGCCTGG AGAAGAAGAC GGCCACTTTT
TRAF2-FL  CAGAGCTCCT GCAGAGGTGC GAGAGCCTGG AGAAGAAGAC GGCCACTTTT

          901                                                   950
TRAF2-TR  GAGAACATTG TCTGCGTCCT GAACCGGGAG GTGGAGAGGG TGGCCATGAC
TRAF2-FL  GAGAACATTG TCTGCGTCCT GAACCGGGAG GTGGAGAGGG TGGCCATGAC

          951                                                  1000
TRAF2-TR  TGCCGAGGCC TGCAGCCGGC AGCACCGGCT GGACCAAGAC AAGATTGAAG
TRAF2-FL  TGCCGAGGCC TGCAGCCGGC AGCACCGGCT GGACCAAGAC AAGATTGAAG

          1001                                                 1050
TRAF2-TR  CCCTGAGTAG CAAGGTGCAG CAGCTGGAGA GGAGCATTGG CCTCAAGGAC
TRAF2-FL  CCCTGAGTAG CAAGGTGCAG CAGCTGGAGA GGAGCATTGG CCTCAAGGAC

          1051                                                 1100
TRAF2-TR  CTGGCGATGG CTGACTTGGA GCAGAAGGTC TTGGAGATGG AGGCATCCAC
TRAF2-FL  CTGGCGATGG CTGACTTGGA GCAGAAGGTC AGGCCCTTCC AGGCGCAGTG
```

FIGURE 4a (continued)

```
         1101                                              1150
TRAF2-TR CTACGATGGG GTCTTCATCT GGAAGATCTC AGACTTTCCC AGGAAGCTCC
TRAF2-FL TGGCCACCGG TACTGCTCCT TCTGCCTGGC CAGCATCCTC AGGAAGCTCC

         1151                                              1200
TRAF2-TR AGGAAGCTGT GGCTGGCCGC ATACCCGCCA TCTTCTCCCC AGCCTTCTAC
TRAF2-FL AGGAAGCTGT GGCTGGCCGC ATACCCGCCA TCTTCTCCCC AGCCTTCTAC

         1201                                              1250
TRAF2-TR ACCAGCAGGT ACGGCTACAA GATGTGTCTG CGTATCTACC TGAACGGCGA
TRAF2-FL ACCAGCAGGT ACGGCTACAA GATGTGTCTG CGTATCTACC TGAACGGCGA

         1251                                              1300
TRAF2-TR CGGCACCGGG CGAGGAACAC ACCTGTCCCT CTTCTTTGTG GTGATGAAGG
TRAF2-FL CGGCACCGGG CGAGGAACAC ACCTGTCCCT CTTCTTTGTG GTGATGAAGG

         1301                                              1350
TRAF2-TR GCCCGAATGA CGCCCTGCTG CGGTGGCCCT TCAACCAGAA GGTGACCTTA
TRAF2-FL GCCCGAATGA CGCCCTGCTG CGGTGGCCCT TCAACCAGAA GGTGACCTTA

         1351                                              1400
TRAF2-TR ATGCTGCTCG ACCAGAATAA CCGGGAGCAC GTGATTGACG CCTTCAGGCC
TRAF2-FL ATGCTGCTCG ACCAGAATAA CCGGGAGCAC GTGATTGACG CCTTCAGGCC

         1401                                              1450
TRAF2-TR CGACGTGACT TCATCCTCTT TTCAGAGGCC AGTCAACGAC ATGAACATCG
TRAF2-FL CGACGTGACT TCATCCTCTT TTCAGAGGCC AGTCAACGAC ATGAACATCG

         1451                                              1500
TRAF2-TR CAAGCGGCTG CCCCCTCTTC TGCCCCGTCT CCAAGATGGA GGCAAAGAAT
TRAF2-FL CAAGCGGCTG CCCCCTCTTC TGCCCCGTCT CCAAGATGGA GGCAAAGAAT

         1501                                              1550
TRAF2-TR TCCTACGTGC GGGACGATGC CATCTTCATC AAGGCCATTG TGGACCTGAC
TRAF2-FL TCCTACGTGC GGGACGATGC CATCTTCATC AAGGCCATTG TGGACCTGAC

         1551                                              1600
TRAF2-TR AGGGCTCTAA CTGCCCCCTA CTGGTGTCTG GGGGTTGGGG GCAGCCAGGC
TRAF2-FL AGGGCTCTAA CTGCCCCCTA CTGGTGTCTG GGGGTTGGGG GCAGCCAGGC

         1601                                              1650
TRAF2-TR ACAGCCGGCT CACGGAGGGG CCACCACGCT GGGCCAGGGT CTCACTGTAC
TRAF2-FL ACAGCCGGCT CACGGAGGGG CCACCACGCT GGGCCAGGGT CTCACTGTAC
```

FIGURE 4a (continued)

```
         1651                                                   1700
TRAF2-TR AAGTGGGCAG GGGCCCCGCT TGGGCGCTTG GGAGGGTGTC GGCCTGCAGC
TRAF2-FL AAGTGGGCAG GGGCCCCGCT TGGGCGCTTG GGAGGGTGTC GGCCTGCAGC

         1701                                                   1750
TRAF2-TR CAAGTTCACT GTCACGGGGG AAGGAGCCAC CAGCCAGTCC TCAGATTTCA
TRAF2-FL CAAGTTCACT GTCACGGGGG AAGGAGCCAC CAGCCAGTCC TCAGATTTCA

         1751                                                   1800
TRAF2-TR GAGACTGCGG AGGGGCTTGG CAGACGGTCT TAGCCAAGGG CTGTGGTGGC
TRAF2-FL GAGACTGCGG AGGGGCTTGG CAGACGGTCT TAGCCAAGGG CTGTGGTGGC

         1801                                                   1850
TRAF2-TR ATTGGCCGAG GGTCTTCGGG TGCTTCCCAG CACAAGCTGC CCTTGCTGTC
TRAF2-FL ATTGGCCGAG GGTCTTCGGG TGCTTCCCAG CACAAGCTGC CCTTGCTGTC

         1851                                                   1900
TRAF2-TR CTGTGCAGTG AAGGGAGAGG CCCTGGGTGG GGGACACTCA GAGTGGGAGC
TRAF2-FL CTGTGCAGTG AAGGGAGAGG CCCTGGGTGG GGGACACTCA GAGTGGGAGC

         1901                                                   1950
TRAF2-TR ACATCCCAGC AGTGCCCATG TAGCAGGAGC ACAGTGGATG GCCTTGTGTC
TRAF2-FL ACATCCCAGC AGTGCCCATG TAGCAGGAGC ACAGTGGATG GCCTTGTGTC

         1951                                                   2000
TRAF2-TR CCTCGGGCAT GACAGGCAGA AACGAGGGCT GCTCCAGGAG AAGGGCCTCC
TRAF2-FL CCTCGGGCAT GACAGGCAGA AACGAGGGCT GCTCCAGGAG AAGGGCCTCC

         2001                                                   2050
TRAF2-TR TGCTGGCCAG AGCAAGGAAG GCTGAGCAGC TTGGTTCTCC CCTCTGGCCC
TRAF2-FL TGCTGGCCAG AGCAAGGAAG GCTGAGCAGC TTGGTTCTCC CCTCTGGCCC

         2051                                                   2100
TRAF2-TR CTGGAGAGAA GGGAGCATTC CTAGACCCCT GGGTGCTTGT CTGCACAGAG
TRAF2-FL CTGGAGAGAA GGGAGCATTC CTAGACCCCT GGGTGCTTGT CTGCACAGAG

         2101                                                   2150
TRAF2-TR CTCTGGTCTG TGCCACCTTG GCCAGGCTGG CTGTGGGAGG GTCTGGTCCC
TRAF2-FL CTCTGGTCTG TGCCACCTTG GCCAGGCTGG CTGTGGGAGG GTCTGGTCCC

         2151                                                   2200
TRAF2-TR ACGCCGCCTC TGCTCAGACA CTGTGTGGGA GGGCACAGCA CAGCTGCGGG
TRAF2-FL ACGCCGCCTC TGCTCAGACA CTGTGTGGGA GGGCACAGCA CAGCTGCGGG
```

FIGURE 4a (continued)

```
         2201                                              2250
TRAF2-TR TAAAGTGTGA GAGCTTGCCA TCCAGCTCAC GAAGACAGAG TTATTAAACC
TRAF2-FL TAAAGTGTGA GAGCTTGCCA TCCAGCTCAC GAAGACAGAG TTATTAAACC


         2251      2262
TRAF2-TR ATTACAAATC TC
TRAF2-FL ATTACAAATC TC
```

```
             1       10         20         30         40         50         60         70         80         90         100
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
h-traf2-fl   MAAASVTPPGSLELLQPGFSKTLLGTKLEAKYLCSACRNVLRRPFQAQCGHRYCSFCLASILSSGPQNCAACVHEGIYEEGISILESSSAFPDNAARREV
h-traf2-tr   MAAASVTPPGSLELLQPGFSKTLLGTKLEAKYLCSACRNVLRRPFQAQCGHRYCSFCLASILSSGPQNCAACVHEGIYEEGISILESSSAFPDNAARREV
 Consensus   MAAASVTPPGSLELLQPGFSKTLLGTKLEAKYLCSACRNVLRRPFQAQCGHRYCSFCLASILSSGPQNCAACVHEGIYEEGISILESSSAFPDNAARREV

             101     110        120        130        140        150        160        170        180        190        200
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
h-traf2-fl   ESLPAVCPSDGCTWKGTLKEYESCHEGRCPLMLTECPACKGLVRLGEKERHLEHECPERSLSCRHCRAPCCGADVKAHHEVCPKFPLTCDGCGKKKIPRE
h-traf2-tr   ESLPAVCPSDGCTWKGTLKEYE------------------------------------------------------------------------------
 Consensus   ESLPAVCPSDGCTWKGTLKEYE

             201     210        220        230        240        250        260        270        280        290        300
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
h-traf2-fl   KFQDHVKTCGKCRVPCRFHAIGCLETVEGEKQQEHEVQWLREHLAMLLSSVLEAKPLLGDQSHAGSELLQRCESLEKKTATFENIVCVLNREVERVAMTA
h-traf2-tr   -FQDHVKTCGKCRVPCRFHAIGCLETVEGEKQQEHEVQWLREHLAMLLSSVLEAKPLLGDQSHAGSELLQRCESLEKKTATFENIVCVLNREVERVAMTA
 Consensus    FQDHVKTCGKCRVPCRFHAIGCLETVEGEKQQEHEVQWLREHLAMLLSSVLEAKPLLGDQSHAGSELLQRCESLEKKTATFENIVCVLNREVERVAMTA

             301     310        320        330        340        350        360        370        380        390        400
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
h-traf2-fl   EACSRQHRLDQDKIEALSSKVQQLERSIGLKDLAMADLEQKVRPFQAQCGHRYCSFCLASILRKLQEAVAGRIPAIFSPAFYTSRYGYKMCLRIYLNGDG
h-traf2-tr   EACSRQHRLDQDKIEALSSKVQQLERSIGLKDLAMADLEQKVLEMEASTYDGVFIWKISDFARKLQEAVAGRIPAIFSPAFYTSRYGYKMCLRIYLNGDG
 Consensus   EACSRQHRLDQDKIEALSSKVQQLERSIGLKDLAMADLEQKVrem#Aqcgdrycifcladia RKLQEAVAGRIPAIFSPAFYTSRYGYKMCLRIYLNGDG

             401     410        420        430        440        450        460        470        480        490        500
             |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
h-traf2-fl   TGRGTHLSLFFVVMKGPNDALLRWPFNQKVTLMLLDQNNREHVIDAFRPDVTSSSFQRPVNDMNIASGCPLFCPVSKMEAKNSYVRDDAIFIKAIVDLTG
h-traf2-tr   TGRGTHLSLFFVVMKGPNDALLRWPFNQKVTLMLLDQNNREHVIDAFRPDVTSSSFQRPVNDMNIASGCPLFCPVSKMEAKNSYVRDDAIFIKAIVDLTG
 Consensus   TGRGTHLSLFFVVMKGPNDALLRWPFNQKVTLMLLDQNNREHVIDAFRPDVTSSSFQRPVNDMNIASGCPLFCPVSKMEAKNSYVRDDAIFIKAIVDLTG

            501
             |
h-traf2-fl   L
h-traf2-tr   L
 Consensus   L
```

FIGURE 4b

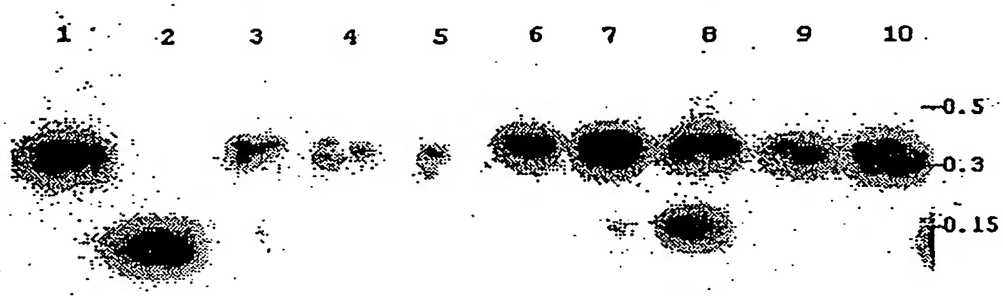

FIGURE 5

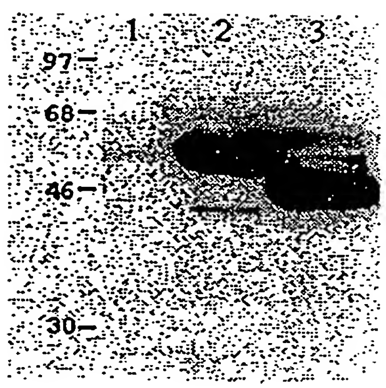

FIGURE 6

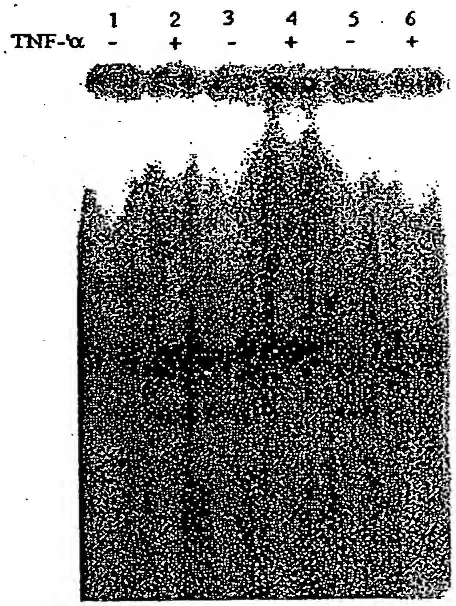

FIGURE 7

Docket No.
P22,816-A USA

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Variants of TRAF2 which Act as an Inhibitor of TNF-Alpha (TNF) Signaling Pathwa

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on April 6, 2000 as United States Application No. or PCT International Application Number PCT/US00/09178

and was amended on ______________________

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(Number)	(Country)	(Day/Month/Year Filed)	☐
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I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional

60/131,940	April 30, 1999
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
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Joseph F. Posillico, Reg. No. 32,290
Mark D. Simpson, Reg. No. 32,942
Joshua R. Slavitt, Reg. No. 40,816
Stephen J. Weed, Reg. No. 45,202
Gene J. Yao, Reg. No. 47,193
Jonathan M. Dermott, Provisional Reg. No. P-48,608
Martin F. Savitzky, Reg. No. 29,699

21

Send Correspondence to: **Patrick J. Kelly**
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2600 Aramark Tower, 1101 Market Street
Philadelphia, PA 19107-2950

Direct Telephone Calls to: *(name and telephone number)*
Patrick J. Kelly - 215-923-4466

Full name of sole or first inventor **George H. Searfoss, III**	5/15/2002 Date
Sole or first inventor's signature George H. Searfoss III	
Residence **Birdsboro, Pennsylvania** PA	
Citizenship **United States of America**	
Post Office Address **16 Valley Drive, Birdsboro, Pennsylvania 19508, USA**	

100

Full name of second inventor, if any **Marco F. Pagnoni**	
Second inventor's signature	Date
Residence **Norristown, Pennsylvania 19403, USA** PA	
Citizenship **United States of America**	
Post Office Address **2029 Sterigere Street, Norristown, Pennsylvania 19403, USA**	

Full name of third inventor, if any Yuri D. Ivashchenko	
Third inventor's signature	Date
Residence Norristown, Pennsylvania 19403, USA PA	
Citizenship United States of America	
Post Office Address 11 Hampton Court, Norristown, Pennsylvania 19403, USA	

3-00

Full name of fourth inventor, if any Kun Guo	
Fourth inventor's signature	Date
Residence Norristown, Pennsylvania 19403, USA PA	
Citizenship United States of America	
Post Office Address 103 Eagle Stream Drive, Apt. #21, Norristown, Pennsylvania 19403, USA	

4-00

Full name of fifth inventor, if any Kenneth L. Clark	
Fifth inventor's signature	Date
Residence Stevenage, Hertfordshire, United Kingdom GBX	
Citizenship United Kingdom	
Post Office Address 34 Granby Road, Stevenage, Hertfordshire SG1 4AS, England	

5-00

Full name of sixth inventor, if any	
Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

[redacted]

Docket No.
P22,816-A USA

Declaration and Power of Attorney For Patent Application

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Direct Telephone Calls to: *(name and telephone number)*
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Full name of sole or first inventor **George H. Searfoss, III**	
Sole or first inventor's signature	Date
Residence **Birdsboro, Pennsylvania**	
Citizenship **United States of America**	
Post Office Address **16 Valley Drive, Birdsboro, Pennsylvania 19508, USA**	

Full name of second inventor, if any **Marco F. Pagnoni**	
Second inventor's signature Marco F. Pagnoni	Date 2-25-02
Residence **Norristown, Pennsylvania 19403, USA**	
Citizenship **United States of America**	
Post Office Address **2029 Sterigere Street, Norristown, Pennsylvania 19403, USA**	

Full name of third inventor, if any	
Yuri D. Ivashchenko	
Third inventor's signature	Date
Residence	
Norristown, Pennsylvania 19403, USA	
Citizenship	
United States of America	
Post Office Address	
11 Hampton Court, Norristown, Pennsylvania 19403, USA	

Full name of fourth inventor, if any	
Kun Guo	
Fourth inventor's signature	Date
Residence	
Norristown, Pennsylvania 19403, USA	
Citizenship	
United States of America	
Post Office Address	
103 Eagle Stream Drive, Apt. #21, Norristown, Pennsylvania 19403, USA	

Full name of fifth inventor, if any	
Kenneth L. Clark	
Fifth inventor's signature	Date
Residence	
Stevenage, Hertfordshire, United Kingdom	
Citizenship	
United Kingdom	
Post Office Address	
34 Granby Road, Stevenage, Hertfordshire SG1 4AS, England	

Full name of sixth inventor, if any	
Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Express Mail Label No. EL663032275US

Docket No.
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Full name of sole or first inventor **George H. Searfoss, III**	
Sole or first inventor's signature	Date
Residence **Birdsboro, Pennsylvania**	
Citizenship **United States of America**	
Post Office Address **16 Valley Drive, Birdsboro, Pennsylvania 19508, USA**	

Full name of second inventor, if any **Marco F. Pagnoni**	
Second Inventor's signature	Date
Residence **Norristown, Pennsylvania 19403, USA**	
Citizenship **United States of America**	
Post Office Address **2029 Sterigere Street, Norristown, Pennsylvania 19403, USA**	

Full name of third inventor, if any **Yuri D. Ivashchenko**	
Third inventor's signature Yu Ivashchenko	Date 20 Feb 2002
Residence **Norristown, Pennsylvania 19403, USA**	
Citizenship **United States of America**	
Post Office Address **11 Hampton Court, Norristown, Pennsylvania 19403, USA**	

Full name of fourth inventor, if any **Kun Guo**	
Fourth inventor's signature	Date
Residence **Norristown, Pennsylvania 19403, USA**	
Citizenship **United States of America**	
Post Office Address **103 Eagle Stream Drive, Apt. #21, Norristown, Pennsylvania 19403, USA**	

Full name of fifth inventor, if any **Kenneth L. Clark**	
Fifth inventor's signature	Date
Residence **Stevenage, Hertfordshire, United Kingdom**	
Citizenship **United Kingdom**	
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Marco F. Pagnoni
Second inventor's signature Date

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Yuri D. Ivashchenko
Third inventor's signature Date

Residence
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United States of America
Post Office Address
11 Hampton Court, Norristown, Pennsylvania 19403, USA

Full name of fourth inventor, if any
Kun Guo
Sep 18, 2002
Date
Fourth inventor's signature
Guo Kun
Residence
Irvine, California 92612
Citizenship
United States of America
Post Office Address
123 Exeter, Irvine, California 92612
125 Exeter

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Kenneth L. Clark
Fifth inventor's signature Date

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United Kingdom
Post Office Address
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Sixth inventor's signature Date

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Mark D. Simpson, Reg. No. 32,942
Joshua R. Slavitt, Reg. No. 40,816
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Martin F. Savitzky, Reg. No. 29,699

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Full name of sole or first inventor **George H. Searfoss, III**	
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Residence **Birdsboro, Pennsylvania**	
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Second inventor's signature	Date
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Citizenship **United States of America**	
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Yuri D. Ivashchenko	
Third inventor's signature	Date
Residence	
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Citizenship	
United States of America	
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Kun Guo	
Fourth inventor's signature	Date
Residence	
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Citizenship	
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Full name of fifth inventor, if any	
Kenneth L. Clark	
Fifth inventor's signature Kenneth L Clark	Date 19 Feb 02
Residence	
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Citizenship	
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Full name of sixth inventor, if any	
Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

SEQUENCE LISTING

<110> Searfoss III, George H.
Pagnoni, Marco F.
Ivashchenko, Yuri D.
Guo, Kun
Clark, Kenneth L.

<120> VARIANTS OF TRAF2 WHICH ACT AS AN INHIBITOR OF TNF-ALPHA (TNFa) SIGNALING PATHWAY

<130> 22816 PCT

<140> As yet unassigned
<141> 2000-04-06

<150> 60/131940
<151> 1999-04-30

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SEQUENCE LISTING

<110> Searfoss III, George H.
Pagnoni, Marco F.
Ivashchenko, Yuri D.
Guo, Kun
Clark, Kenneth L.

<120> VARIANTS OF TRAF2 WHICH ACT AS AN INHIBITOR OF TNF-ALPHA (TNFa) SIGNALING PATHWAY

<130> 22816 PCT

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<141> 2000-04-06

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<151> 1999-04-30

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Val Pro Cys Arg Phe His Ala Ile Gly Cys Leu Glu Thr Val Glu Gly
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Glu Lys Gln Gln Glu His Glu Val Gln Trp Leu Arg Glu His Leu Ala
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